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PCR and FISH Analysis of a Ring Y Chromosome

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A newborn male infant presented with midshaft hypospadias, chordee, and undescended left testis. Both gonads lacked the tunica albuginea and appeared to be adjacent to structures resembling fallopian tubes. On biopsy, there was marked dysgenesis of both gonads, with a paucity of testicular tubules and foci of ovarian-like stroma. Peripheral blood karyotype was 46,X,mar(Y) [39]/45,X [5]. Right gonadal biopsy material showed the same mosaicism but with a higher proportion of 45,X cells (46%). PCR and FISH analyses with primers/probes from different Yp, Yq, and Ycen loci defined the structure of the marker Y as a probable complex ring with breakpoints in Yq11.21 (very close to the centromere) and in Yp11.32 (the pseudoautosomal region). Based on the phenotype and the laboratory findings, the prognosis given to the patient was for short stature and azoospermia without an increased risk for gonadoblastomas. Am. J. Med. Genet. 69:171–176, 1997.

KEY WORDS: Y chromosome; ring Y; FISH; PCR; mosaicism; hypospadias; dysgenetic testes; Sertoli-cell only

INTRODUCTION

Normal male development depends on the presence of the Y chromosome in humans and other mammalian species. This chromosome is involved in numerous structural abnormalities [Hsu, 1994], and the phenotype of individuals with Y chromosome abnormalities depends on the deleted genes.

During routine cytogenetic studies, we karyotyped the blood and gonadal tissue of a phenotypically male infant with hypospadias, left cryptorchidism and gonadal dysplasia. In both tissues, the karyotype was mosaic, 45,X/46,X,mar(Y) with a lower proportion of 45,X cells in the blood (11%) than in the gonad (46%). Given the male phenotype, it was presumed that the marker was of Y origin and that the risk for gonadoblastomas was not increased. Given the very small size of the marker chromosome, the purpose of this study was (1) to verify the origin and to determine the structure of the marker and the approximate position of the breakpoint(s) by using molecular cytogenetic techniques and (2) to establish a prognosis for this child based on genes present or absent in the marker.

CLINICAL REPORT

The patient was a newborn infant with no significant abnormalities on physical examination, other than abnormalities of the genitalia. The parents were nonconsanguineous and the family history was unremarkable. The pregnancy was uncomplicated. At age 8 months the patient's length was 65.2 cm (5th centile) and the weight was 6.94 kg (5th centile). The infant had a normally formed scrotum and adequate size of phallus (3.5 cm). There was marked chordee and midshaft hypospadias. The right testis was in the scrotum, and the left testis was in the inguinal canal. Both testes were normal in size and had a normal consistency on palpation. Laboratory tests showed normal testosterone response to HCG stimulation.

At age 10 months, the boy's hypospadias was repaired, and a left orchiopexy was performed. Tubular structures resembling fallopian tubes were found in the vicinity of both gonads. Biopsies of both testes showed dysgenesis, with marked decrease in the number of seminiferous tubules, thickened testicular basement membrane, and foci of ovarianlike stroma, but oocytes or primordial follicles were not seen. Germ cells were absent. Some calcification and hemosiderin deposition was seen in the left gonad.

MATERIALS AND METHODS

Cytogenetic Studies

Metaphases were prepared from PHA-stimulated peripheral blood lymphocytes and from cells grown from the right testis biopsy material. Colcemid treatment, harvesting and slide preparation were performed ac-

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cording to standard laboratory procedures. GTG-banded metaphases were analyzed at the 550 band level.

Fluorescence In Situ Hybridization (FISH) Analysis

For FISH analysis, plasmid probes Y-190 (DYZ5 locus, Yp11.1-11.2), pDP97 (DYZ3 locus, Y centromere) and YAC clones yOX28 and yOX44 (CSF2RA gene locus in the Yp pseudoautosomal region, Yp11.32) were used (ATCC, Rockville, Maryland). Cosmid probe T1 (pseudoautosomal region,Yp11.32) was a gift from Dr. G. Rappold, Heidelberg University, Germany. The X centromere probe DNA was obtained by PCR amplification of normal genomic DNA using published primer sequences (ATCC, Rockville, Maryland): primers X-3A and X-4A (defining the DXZ1 locus). Total human telomere probe was generated by PCR, using the telomere repeat oligonucleotides $(TTAGGG)\rightarrow_5$ and $(CCCTAA)\rightarrow_5$ in the presence of Dig-11-dUTP but without a template [Ijdo et al., 1991]. DNA was prepared and labeled by nick translation (or PCR for the X-centromere) using differently labeled nucleotides (Table I). Before using FISH, slides were pretreated with pepsin; 5–10 ng labeled cosmid or plasmid probes or 150 ng labeled total yeast DNA was used for each hybridization, with 500× Cot1 DNA (Gibco BRL). Slides and probes were denatured together at 75°C for 2 minutes; hybridization took place overnight, at 37°C. Direct labeled probes were visualized directly under the fluorescence microscope. Others were detected indirectly with avidin conjugated with FITC (Boehringer) or AMCA (Vector) for biotin, or antibodies conjugated with Cy3 (Accurate) for digoxigenin (Table I). Microscopic analysis of FISH images was done using an Aristoplan fluorescence microscope (Leica) equipped with appropriate filters. For a better resolution of simultaneously hybridized probes, a Zeiss Axiophot microscope connected to a cooled charged-coupled device (CCD) camera (Photometrics) and a software package developed by Oncor, Inc (Gaithersburg) were used. Separate gray images of the three probes (red, green, and blue) were taken using individual FITC, rhodamine, and DAPI filters. Acquired images were pseudocolored and superimposed by the computer to give the final image.

Genomic DNA Preparation

Genomic DNA from fibroblasts and blood cells was prepared using SDS and proteinase K, following standard laboratory procedures.

TABLE I. List of Y-Probes and Labeling Systems Used for Each of Them

Probe	Label	Detection	Color
pY-190	Cy3-6-dCTP	Direct	Red
	DNP-11-dUTP	FITC-Ab	Green
pDP97	Cy3-6-dCTP	Direct	Red
	FITC-12-dUTP	Direct	Green
	Biotin-11-dUTP	FITC-avidin	Green
		AMCA-avidin	Blue
yOX28, yOX44 and CosT1	Dig-11-dUTP	Cy3-Ab	Red
PCR telomere	Dig-11-dUTP	Cy3-Ab	Red
X centromere	Biotin-11-dUTP	FITC-avidin	Green

PCR Reactions

Primers sY14 (SRY gene locus) and sY81 (locus DYS271 on Yq) [Vollrath et al., 1992] (Fig. 4a) were used to test the presence or absence of the corresponding loci on the Y chromosome. In each reaction 100–150 ng DNA (patient or control) was used in a 9600 Thermocycler (Perkin Elmer Cetus), denaturing for 40 seconds at 94°C, annealing for 30 seconds at 54°C and extending for 90 seconds at 68°C (35 cycles). Then 6–7 µl of PCR products was separated on a 1.5% agarose gel and visualized after staining with ethidium bromide.

RESULTS

Cytogenetic Analysis

We analyzed 44 metaphases from the peripheral blood and 50 metaphases from the testis cultures. In both tissues 46,X,mar(Y)/45,X mosaicism was present, but in different proportions: 11% in the peripheral blood and 46% in the right testis for the 45,X line. The marker chromosome (Fig. 1) was very small and appeared to be a ring or a small isochromosome.

PCR Analysis

DNA from leukocytes of the patient plus a positive (male) and a negative (female) control DNA were tested in separate PCR reactions using two primer pairs, sY81 (locus DYS271) on Yq11.21 and sY14 (SRY gene locus) on Yp11.3 near the Yp pseudoautosomal boundary. Results are shown in Figure 2. The patient's DNA showed the presence of the previously described 472 bp amplification fragment of the SRY gene locus. However, only the positive control male DNA showed amplification of the 209 bp product of primer pair sY81, suggesting loss of this locus in the patient and a breakpoint on Yq probably less than 1 mb distance from the centromere.

FISH Analysis

FISH was performed with the Y-centromere probe (Fig. 3a) in order to verify that the marker was derived from a Y chromosome. With this probe, a signal was seen in 45 of 100 cells counted, confirming the mosaicism found after G-banding.

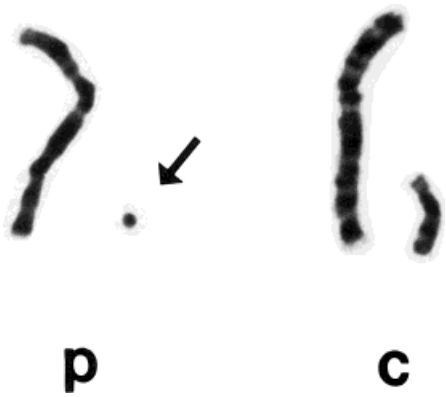

Fig. 1. Comparison of the sex chromosomes of the proband (p) with the sex chromosomes of a normal male (c), showing the small size of the marker Y (arrow).

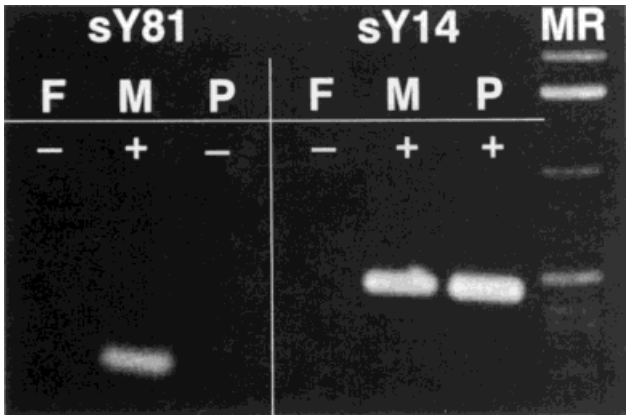

Fig. 2. PCR amplification using primer pairs sY81 (**lanes 1–3**) and sY14 (**lanes 4–6**). Lanes 1 and 4 (negative control, female genomic DNA) show no amplification. Lanes 2 and 5 (positive control, normal male genomic DNA) show both amplification products. Patient DNA shows amplification of the SRY gene locus (primer pair sY14, lane 6) but no amplification product for the primer pair sY81 (lane 3). M = standard DNA size marker (1 kb ladder, Gibco BRL).

Probe Y-190 detects a repetitive locus (DYZ5), approximatively 1 Mb from the centromere on Yp. The DNP-labeled Y-190 probe showed hybridization signal on the marker similar to the signal of the pDP97 (DYZ3) probe (Fig. 3a,b), confirming the presence of this Yp region. Interphase nuclei showed only one signal for each of these probes (data not shown), indicating that the centromere and this Yp region probably were not duplicated, and the marker was not an isochromosome.

To determine the length of the Yp segment, the marker was further characterized by FISH with YACs yOX28 and yOX44 and cosmid T1 (pseudoautosomal region on Yp, Xp). The marker chromosome was negative for all three, indicating that it had one breakpoint on distal Yp, as well as one on proximal Yq. This result suggested that the marker might have been a ring Y chromosome with most of Yq and the tip of Yp deleted.

To confirm this, FISH was performed with a total human telomere probe. As seen in Figure 3c, the telomere probe showed a single, strong hybridization signal on the marker Y. Figure 3a–d shows the simultaneous hybridization of the biotinylated Y-centromere probe (blue

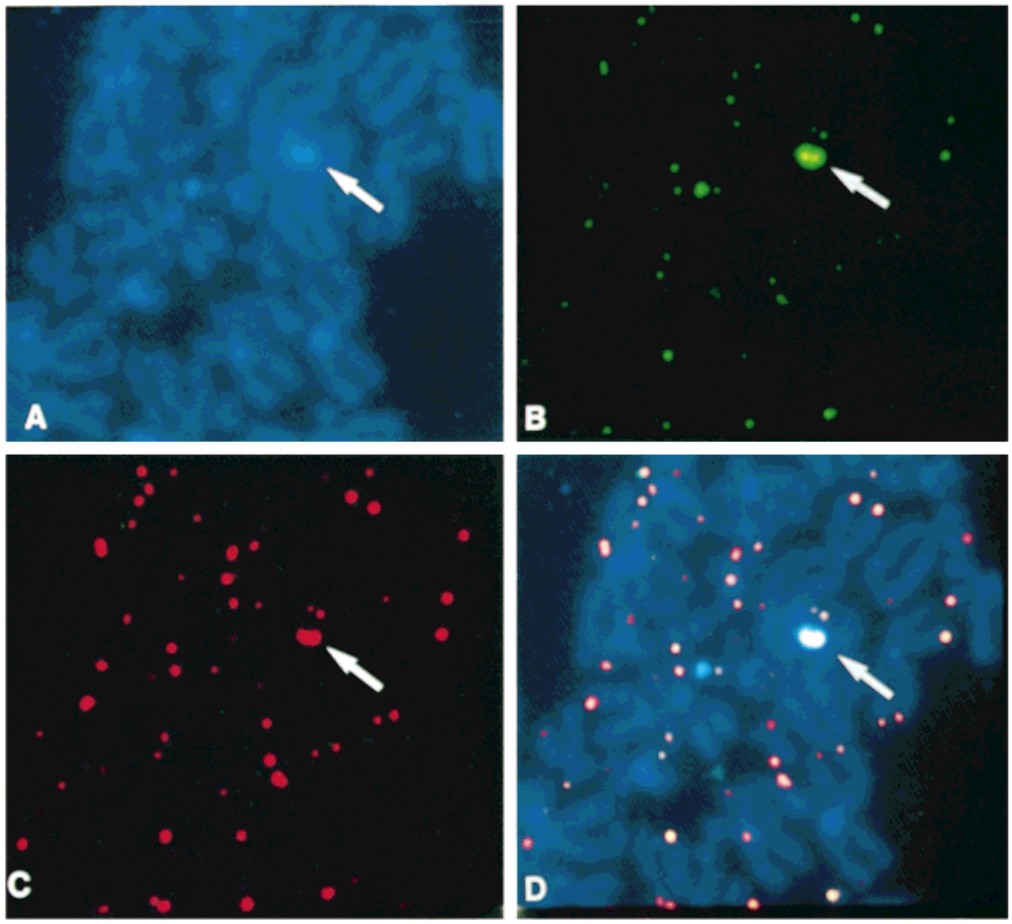

Fig. 3. Simultaneous hybridization with (**a**) the biotinylated Y centromere probe detected blue with AMCA, (**b**) the DNP-labeled Yp probe Y-190 detected green with FITC, and (**c**) the digoxigeninylated total human telomere probe detected red with Cy3. **d:** Computer-combined images a, b, and c. All marker chromosomes analyzed showed only one telomeric signal, indicating that the marker was probably a ring.

with AMCA) the DNP-labeled Y-190 probe (green with FITC) and the digoxigeninylated total human telomere probe (red with Cy3) on a marker chromosome. Both pDP97 (Y centromere) and Y-190 probes gave very specific signals, with no background (Fig. 3a,b). The telomere signals (Fig. 3c) consistently overlapped the centromere and Yp probe signals. The extra green signals in Figure 3b are due to partial leakage of the red-orange emission of the Cy3-detected telomere signals through the FITC filter. When images were overlapped (Fig. 3d) the combination of the basic red, green and blue yielded the white color. These observations confirmed that the marker was a ring chromosome, with some of the telomeric sequences preserved at the breakpoint site(s).

DISCUSSION

Karyotypic analysis of blood and testis tissue of a newborn infant with abnormalities of the genitalia showed 46,X,mar(Y)/45,X mosaicism, with a higher proportion of 45,X cells (46%) in the right gonad than in the blood (11%). PCR and multicolor FISH analyses were used to confirm that the marker was of Y origin and to define its structure as a complex ring (Fig. 2 and 3). FISH with probes for the Y-centromere (pDP97), a proximal Yp locus (pY-190) and the Yp-Xp pseudoautosomal region (yOX28, yOX44, cosT1) showed that the marker had a breakpoint in distal Yp and was not a dicentric. PCR with primers for the SRY gene locus on Yp and for the proximal Yq locus DYS271 showed that the Yp breakpoint was distal to the SRY gene and that there was a second breakpoint between the Y centromere and the DYS271 locus (deletion interval 5A) [Vollrath et al., 1992]. Relative position of these probes on the Y chromosome is depicted in Figure 4a.

FISH with a total human telomere probe showed only one hybridization signal on each marker analyzed. The telomeric signal(s) was always inside of the mass of the marker chromosome, indicated that the marker probably was not an isochromosome or a dicentric, but a ring (Fig. 4b). Presence of a ring chromosome may explain both the presence of mosaicism and the differing proportions of mosaicism in blood and testes, as a ring chromosome may be unstable and may be lost in mitosis [Fang et al., 1995].

There are several possible mechanisms of formation of this ring chromosome. One possibility is that an initial break occurred in either the Yp or the Yq arm, forming a "sticky end". The "sticky end" must be healed promptly [Matsumoto et al., 1987; Hastie and Allshire, 1989; Day et al., 1993; Meltzer et al., 1993]. This probably occurred through the fusion of the broken DNA end with the opposite telomere region of the Y chromosome and formation of a ring structure. As ring chromosomes may be unstable [Fang et al., 1995], the initial ring chromosome may have lost additional DNA on one side of the telomeric sequences, leading to a smaller and possibly more stable Y ring. This could explain the existence of telomeric sequences in the ring structure and the loss of DNA sequences in both Yp and Yq. As Yq is almost completely absent, it is also possible that the initial ring lost more and more of Yq in a stepwise man-

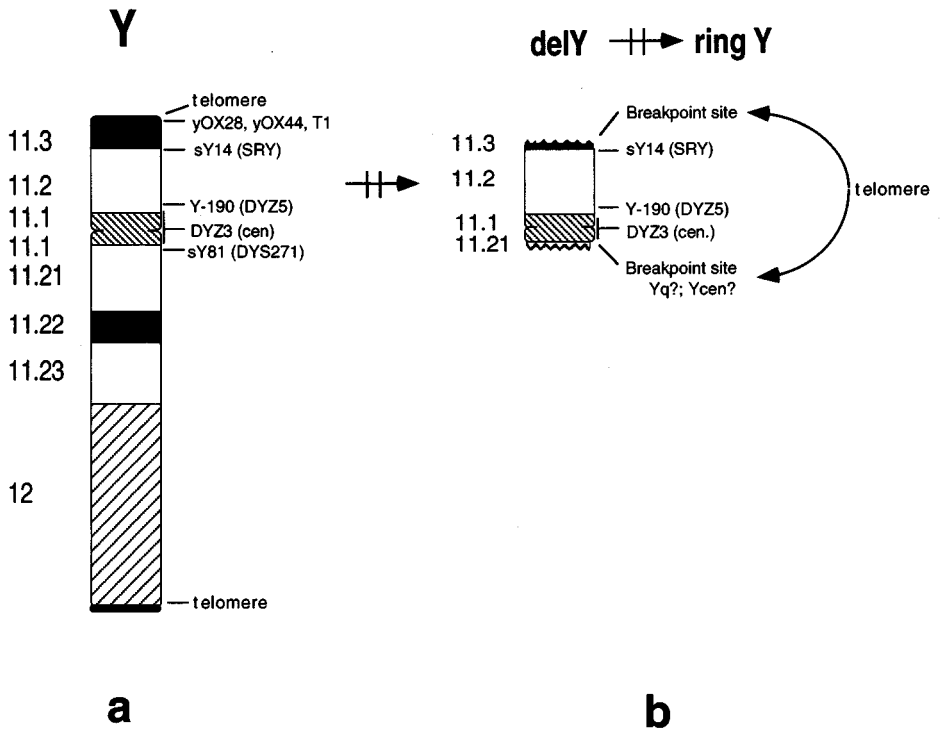

Fig. 4. **a:** Relative positions on the Y chromosome of the probes used for FISH and PCR studies. **b:** Probes that were positive on the marker, either by FISH or by PCR. The semicircular arrow indicates probable formation of a ring Y chromosome.

ner, until it reached the final length. Another possible mechanism of formation of the ring is through initial fusion of the two telomeric ends of the Y and subsequent loss of Y sequences on both sides of the telomeric fusion point. Such fusions previously have been shown to occur in ring formation [Park et al., 1992; Pezzolo et al., 1993]. Although telomeric sequences were not always detected in ring chromosomes [Hou et al., 1992], some previous FISH studies using a telomere repeat probe detected at least one telomere sequence in each ring analyzed [Park et al., 1992; Pezzolo et al., 1993].

The molecular studies of this patient provide a basis for predicting prognosis, especially regarding the risk of developing gonadoblastomas and infertility. The male phenotype of the patient indicates the presence of the SRY gene locus, and that this gene was active during embryogenesis [Goodfellow and Lovell-Badge, 1993; Lovell-Badge, 1993]. The SRY gene is essential for the differentiation of the medulla of the primordial gonads (indifferent gonads) into seminiferous tubules and testis (about 6th week) [Ferguson-Smith, 1992; Lovell-Badge, 1992; Mittwoch, 1992]. Mutations of the SRY gene have been shown to cause altered phenotypes in genetic males, ranging from various grades of dysgenetic testes to a complete lack of testicular tissue and a female phenotype (46,XY females) [Weckworth et al., 1988; Haaf and Schmid, 1990; Cooper et al., 1991; Fechner et al., 1992; Jager et al., 1992; Lindgren et al., 1992; Affara et al., 1993; Mendez et al., 1993; Mittwoch, 1993; Vilain et al., 1993; Sugarman et al., 1994]. In cases with mosaicism of the Y chromosome (as in this patient), in which not all cells carry the SRY gene locus, the higher the proportion of cells without a functional SRY, the more dysgenetic the gonads appear. Dysgenetic gonads have a decreased density of seminiferous tubules (containing Sertoli cells, which produce müllerian inhibiting substance [MIS]) and possibly also of the Leydig cell population (testosterone production), hence an increased severity of the malformations of the genitalia and preservation of female genital tract structures. These observations correlate well with the anomalies in this patient. The gonads are dysgenetic, with few seminiferous tubules, ovarian-like stroma, and both gonads have Fallopian tube structures in their vicinities. The high proportion of 45,X cells in the gonads are consistent with this pathology. Decreased testosterone and/or MIS production during early development might have caused the malformations of the external genitalia and the preservation of the fallopianlike structures, respectively.

Other genes, not yet cloned but postulated to exist near the centromere region, especially on Yq, might influence the phenotype of this patient, as the Yq breakpoint in the marker is very close to the centromere. Based on the short stature of most males with deletions of proximal Yq, the GCY gene locus is postulated on Yq11 [Ogata and Matsuo, 1993; Salo et al., 1995]. Another stature gene is postulated to exist in the Yp pseudoautosomal region [Henke et al., 1991]. It is possible that one or both of these genes is deleted in this patient and will affect his stature. Currently, his height is at the 5th percentile. The GBY gene is believed to be located close to the Y centromere [Tsuchiya et al., 1995], and patients with Ullrich-Turner syndrome, carrying pericentromeric Y sequences, are at high risk for developing gonadoblastomas [Page, 1987, 1994; Krasna et al., 1992; Lukusa et al., 1992; Nagafuchi et al., 1992; Barbosa et al., 1995]. However, current data support the observation that there is no increased risk for this tumor in phenotypically male patients with deletions of various regions of the Y chromosome [Chapman et al., 1990; De Arce et al., 1992; Erhan et al., 1992; Tharapel et al., 1992; Macera et al., 1994].

Male patients with deletions of the entire Yq are azoospermic; one or two AZF genes have been postulated to exist in the Yq euchromatin [Vogt et al., 1992; Kobayashi et al., 1994]. This fact strongly suggests that our patient will have azoospermia.

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